SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS DTIC FILE COPY
2a. SECURITY CLASSIFICATION AUTHORITY **AD-A213 054**	3. DISTRIBUTION / AVAILABILITY OF REPORT
	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION DIVISION of CD&I, WRAIR Washington, DC 20307-5100	6b. OFFICE SYMBOL (If applicable) SGRD-UWF-H
7a. NAME OF MONITORING ORGANIZATION Walter Reed Army Institute of Research Washington, DC 20307-5100
6c. ADDRESS (City, State, and ZIP Code) Washington, DC 20307-5100	7b. ADDRESS (City, State, and ZIP Code) Washington, DC 20307-5100
8a. NAME OF FUNDING / SPONSORING ORGANIZATION Ft. Detrick, Frederick, MD	8b. OFFICE SYMBOL (If applicable)
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) US Army Med Rsch & Dev Command Ft. Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
UNCLAS-Shigella flexneri Invasion Plasmid Antigens B & C: Epitope Location and Characterization With Monoclonal Antibodies.

12. PERSONAL AUTHOR(S)
Mills, J A; Buysse, J; and Oaks, E.

13a. TYPE OF REPORT	13b. TIME COVERED FROM ____ TO ____	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

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20 DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION	
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED	
22a NAME OF RESPONSIBLE INDIVIDUAL Jonathan A Milss	22b TELEPHONE (Include Area Code) 202-576-1236	22c OFFICE SYMBOL SGRD-UWF-H

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

INFECTION AND IMMUNITY, Nov. 1988, p. 2933–2941
0019-9567/88/112933-09$02.00/0

Vol. 56, No. 11

Shigella flexneri Invasion Plasmid Antigens B and C: Epitope Location and Characterization with Monoclonal Antibodies

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Received 7 June 1988/Accepted 9 August 1988

Invasion plasmid antigens B (IpaB) and C (IpaC) are associated with the ability of shigellae to invade cultured mammalian cells. Monoclonal antibodies against IpaB and IpaC polypeptides were produced and used in a whole-cell enzyme-linked immunosorbent assay to show that both IpaB and IpaC polypeptides were exposed on the surface of virulent shigellae. Moreover, these surface epitopes were shown to be highly conserved among different serotypes of *Shigella* spp. and enteroinvasive *Escherichia coli*. Cross-reactive epitopes were not found on noninvasive *Shigella* strains or on other enteric bacteria including *Salmonella*, *Yersinia*, *Campylobacter*, *Vibrio*, and *Aeromonas* spp. and various pathogenic strains of *E. coli*. The monoclonal antibodies were used in competitive binding assays to define three unique epitopes of the IpaB polypeptide and four unique epitopes of the IpaC polypeptide. Epitope locations and their corresponding DNA-encoding regions were defined by examining the IpaB and IpaC products expressed by λgt11 recombinants and by constructing a genetic map of the insert DNAs of these recombinants. Three IpaB epitopes (2F1, 1H4, 4C8) were found to be encoded on three contiguous DNA regions comprising a 700-base-pair (bp) segment that corresponded to the amino-terminal end of the IpaB polypeptide. Similarly, a 640-bp DNA segment that corresponded to the amino-terminal end of the IpaC polypeptide was found to encode three clustered IpaC epitopes (5H1, 9B6, 5B1). Approximately 50 bp downstream from this region a fourth IpaC epitope-encoding region (2G2) was found. The effect of the monoclonal antibodies on plaque formation by virulent *Shigella flexneri* on a monolayer of cultured mammalian cells (a sensitive measure of invasiveness) was determined. Only the IpaB-specific monoclonal antibody 2F1 was able to reduce the plaque-forming capacity by >50%, suggesting that this epitope of the IpaB polypeptide is involved in the invasion process.

Bacillary dysentery is caused by all members of the genus *Shigella* (*S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*) and also enteroinvasive *Escherichia coli* (EIEC). To cause dysentery, *Shigella* spp. and EIEC must be able to recognize, invade, and multiply within epithelial cells of the colon (17). Both the bacteria and host cell play an active role in the invasive process (6, 7), with the host cell actively engulfing the bacteria which in turn escape from the phagosome by a bacteria-mediated digestion of the phagosomal membrane (29). Once in the cytoplasm, intracellular bacterial multiplication and, subsequently, host cell necrosis occur. Although chromosomal genes are necessary for the production of many components (e.g., lipopolysaccharide, Shiga toxin, Sereny reaction) of the virulent phenotype (reviewed by D. J. Kopecko, M. Venkatesan, and J. M. Buysse, *in* M. Farthing and G. Keusch, ed., *Enteric Infection: Mechanisms, Manifestations, and Management*, in press), the invasive phenotype is encoded by a large, 120- to 140-megadalton (MDa), nonconjugative plasmid (10, 16, 27, 28). The invasion plasmid contains highly conserved genes which are present in all virulent *Shigella* spp. and EIEC (26). Among the conserved genes are four *ipa* genes encoding the IpaA (previously termed polypeptide a; 78 kDa), IpaB (b; 57 kDa), IpaC (c; 43 kDa), and IpaD (d; 39 kDa) polypeptides (4, 8, 20, 36). The four genes are located on a 7.0-kilobase (kb) DNA segment of the *S. flexneri* serotype 5 strain M90T-W invasion plasmid (1). In turn, the *ipa* genes comprise a portion of a 37-kb fragment which, when cloned into a cosmid vector, restores HeLa cell invasiveness to a *Shigella* strain lacking the invasion plasmid (20).

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Polypeptides IpaA, IpaB, IpaC, and IpaD, along with a 140-kDa polypeptide antigen, also function as important immunogens in that they are the dominant protein antigens inducing a serum immune response during infection in humans and monkeys (23). Although these five polypeptides are only minor components of the total complement of bacterial proteins, immunoblots indicate that in many cases the only detectable serum antibody response is against these invasion plasmid antigens, with polypeptides IpaB and IpaC being particularly immunogenic (23). The significance of the serum antibody response to the Ipa polypeptides is not clear at this time, but it could be a determining factor in the limitation of shigellae to the gut, with only rare systemic spread. The presence of antibody against the Ipa polypeptides in the gut has not been determined directly, but cultured colonic biopsies, obtained from infected monkeys, have been shown to produce immunoglobulin A (IgA) antibodies against IpaB and IpaC polypeptides (5).

The existing genetic and serological data strongly suggest that polypeptides IpaB and IpaC are closely associated with the invasive phenotype. Understanding the biological role(s) of the Ipa polypeptides will require specific probes to monitor functional and biochemical properties of these polypeptides. This study describes the production and characterization of specific monoclonal antibodies (MAbs) against three epitopes of the *ipaB* and four epitopes of the *ipaC* gene products. These antibody molecules were used to map the corresponding DNA-encoding region of each IpaB and IpaC epitope and were also used to assess the effect of antibody on the invasive capacity of *S. flexneri*.

(This work was done in part by J.A.M. in partial fulfillment of the requirements for the Master of Science degree at

TABLE 1. Virulence specificity of MAbs against shigella invasion plasmid-encoded IpaB and IpaC polypeptide antigens

Species	Serotype	Strain	Source[a]	Virulence	MAb reactivity
S. flexneri	1b	M25-8	WRAIR	+	+
	1b	M25-8A	WRAIR		
	2a	2457T	WRAIR	+	+
	2a	2457O	WRAIR		
	3a	J17B	WRAIR	+	+
	3a	2783-71	CDC		
	4b	M76-39	WRAIR	+	+
	4a	1862-71	CDC		
	5	M90T-W	WRAIR	+	+
	5	M90T-A_3	WRAIR		
	6	2924-71	CDC	+	+
	6	CCHO60A	WRAIR		
S. dysenteriae	1	JVA70	WRAIR		+
	1	JVA70A	WRAIR		
	2	CG1314	JHU	+	+
	2	CG2002	JHU	+	+
	3	H105 81	JHU	+	+
	4	CG1768	JHU	+	+
	9	H77 82	JHU	+	+
S. boydii	4	CG1156	JHU	+	+
	4	ST227-C	JHU	+	+
S. sonnei	Form I	53G	WRAIR	+	+
	Form II	53G	WRAIR		
		ERO71A	JHU	+	+
		PA232	JHU	+	+
EIEC	O124	M41-63T	WRAIR	+	+
	O136	10673 70	WRAIR	+	+
	O143	4608-58	WRAIR	+	+
	O143	4608-58A	WRAIR		
	O144	1624-56	WRAIR	+	+

[a] WRAIR, Walter Reed Army Institute of Research; CDC, Centers for Disease Control; JHU, The Johns Hopkins University School of Medicine.

[b] Strains indicated as virulent (+) bound Congo red dye, invaded HeLa cells, and tested Sereny positive; avirulent strains (−) did not bind Congo red dye, did not invade HeLa cells, and tested Sereny negative.

[c] Reactivity determinations were based on whole-cell ELISA. Bacterial cells were attached to plates and incubated with MAb. The MAbs were detected with alkaline phosphatase-conjugated goat anti-mouse IgG. A positive test had an A_{405} of ≥0.30 within 60 min. The bacterial strains screened either reacted with all of the MAbs (+) or none of the MAbs (−).

The Catholic University of America, Washington, D.C., 1988.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Shigella* and EIEC strains used in this study are listed in Table 1. Bacterial cultures were routinely grown in Penassay broth or on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) at 37°C. *Shigella* and EIEC strains that bound Congo red dye (21), invaded HeLa cells (6), and elicited keratoconjunctivitis in guinea pigs (30) were considered virulent.

Immunization of mice. A preparative isoelectric focusing (PIEF) procedure described previously (8) was used to partially purify the IpaB and IpaC polypeptides from *S. flexneri* serotype 5 strain M90T-W. PIEF fractions containing IpaB and IpaC polypeptides, as determined by immunoblotting against convalescent monkey antisera, were pooled together and used to immunize female BALB/c mice (3 to 5 months old; Jackson Laboratory, Bar Harbor, Maine). The mice were initially immunized subcutaneously with 100 μg of PIEF antigen mixed with an equal volume of complete Freund adjuvant. Two booster immunizations were given at 4-week intervals (intraperitoneally followed by intravenously); PIEF antigen (100 μg of protein per mouse) without adjuvant was used in each booster injection. The protein concentration of the PIEF antigen was measured by the Bio-Rad protein assay (2), using bovine serum albumin as the standard.

Production, screening, and selection of hybridomas. Three days after the final immunization, spleen cells from immunized BALB/c mice were fused with hypoxanthine-guanine phosphoribosyltransferase-deficient X63-Ag8.653 myeloma cells (13) as described previously (14, 15). Fusions were performed at a ratio of 10 spleen cells to 1 myeloma cell in the presence of polyethylene glycol 1000 (J. T. Baker Chemical Co., Phillipsburg, N.J.). Hybridomas whose culture supernatants tested positive in the enzyme-linked immunosorbent assay (ELISA) against the PIEF antigen were also tested in an immunoblot assay against whole-cell sodium dodecyl sulfate (SDS) lysates of *S. flexneri* serotype 5 strain M90T-W (virulent) and strain M90T-A_3 (avirulent). The hybridomas producing virulence-specific antibodies (i.e., reactive only with M90T-W) were cloned twice by limiting dilution. Ascites fluids were prepared in pristane-primed BALB/c mice by intraperitoneal injection of 10^6 cloned hybrid cells per mouse. The immunoglobulin subclass of each MAb was determined by Ouchterlony analysis using hybridoma culture supernatants and rabbit antisera specific for the IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA subclasses of mouse immunoglobulins (Miles Laboratories, Elkhart, Ind.).

ELISA. The ELISA used to screen hybridoma culture supernatants consisted of 96-well polystyrene plates (Costar 3590; Costar, Cambridge, Mass.) coated with PIEF antigen (1 μg per well) diluted in 10 mM phosphate-buffered saline (PBS) (pH 7.4). The PIEF antigen was allowed to attach overnight at 4°C; unbound PIEF antigen was decanted. A filler consisting of 2% casein and 0.1% sodium azide in 10 mM Tris-buffered saline solution (pH 7.4) was incubated in the wells for 30 min at 25°C and then decanted. Hybridoma culture supernatants were added to the antigen-coated wells and incubated for 2 h at 37°C, followed by three rinses with PBS containing 0.05% Tween-20. Alkaline phosphatase-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Md.), diluted 1:500 in casein filler, was added for 1 h at 25°C, followed by two more rinses with PBS–0.05% Tween-20. *p*-Nitrophenylphosphate (1 mg/ml in diethanolamine buffer, pH 9.8) was added, and after 60 min at 25°C the A_{405} was determined on a Microelisa Auto-Reader (Dynatech Laboratories, Inc., Alexandria, Va.).

The reactivity of the MAbs with *Shigella* spp., EIEC, and other enterics was determined by a whole-cell ELISA. Overnight bacterial cultures collected by centrifugation and suspended in PBS were added to 96-well plates (10^8 cells per well) and allowed to attach for 1 h at 25°C; unattached cells were decanted. After blocking with casein filler, hybridoma ascites fluids (1:300 in casein filler) were incubated with bacterial cell-coated plates for 2 h at 37°C, followed by washing and development as described above. Titers for each MAb were also determined in the whole-cell ELISA against both *S. flexneri* M90T-W and M90T-A_3. The cutoff point for a positive test was an A_{405} of ≥0.30 at 60 min.

Immunoblotting procedure. *Shigella* polypeptides of whole-cell SDS lysates (8) were separated on gels consisting of 13 or 18% acrylamide cross-linked with *N,N'*-diallytartardiamide in a discontinuous SDS-polyacrylamide gel electrophoresis system using Laemmli buffers (18) and electroeluted onto nitrocellulose (Bio-Rad Laboratories, Richmond, Calif.) as previously described (3, 23). After the nitrocellu-

lose was blocked with casein filler for 30 min at 25°C, nitrocellulose strips corresponding to the lanes of the separated polypeptides were incubated overnight at 4°C in one of the following primary antibody solutions: (i) undiluted hybridoma culture supernatants; (ii) hybridoma ascites fluids diluted 1:1,000 in casein filler; or (iii) convalescent monkey antiserum diluted 1:300 in casein filler. Next, the strips were washed, as previously described (23), and incubated at 25°C with alkaline phosphatase-conjugated goat anti-mouse IgG or alkaline phosphatase-conjugated staphylococcal protein A (Kirkegaard and Perry Laboratories) diluted 1:500 in casein filler. After 1 h of incubation, the strips were washed and exposed to the phosphatase substrate (2 mg of fast red TR salt and 1 mg of naphthol AS-MX phosphate [Sigma Chemical Co., St. Louis, Mo.] per ml in 50 mM Tris, pH 8.0) for 30 min. A red precipitate formed on the strips in areas containing alkaline phosphatase activity (31).

Because of inherent background antibodies against many of the *Shigella* polypeptides in various antisera including the conjugates (goat anti-mouse; unpublished observations), a direct immunoblot assay, using iodinated MAbs, was used to confirm the specificities of the MAbs. Ammonium sulfate-precipitated MAbs were labeled with carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.) by the chloramine-T method (11). After the nitrocellulose strips containing virulent or avirulent shigella lysates were blocked with casein, they were incubated with approximately 10^6 cpm of ^{125}I-labeled MAb for 3 h at 25°C. Other nitrocellulose strips that had been incubated with convalescent monkey antiserum were washed and probed with 10^6 cpm of ^{125}I-labeled staphylococcal protein A (Amersham Corp.) for 3 h at 25°C. Finally the strips were washed, air dried, and exposed to X-ray film (Kodak Blue Brand; Eastman Kodak Co., Rochester, N.Y.).

Determination of unique epitopes by a competitive binding assay. Wells of polyvinylchloride microtiter plates (Dynatech Laboratories) were coated with whole bacterial cells (*S. flexneri* M90T-W) and blocked with casein filler as described in the ELISA protocol. The unlabeled blocking antibodies, which had been ammonium sulfate precipitated and resolubilized in PBS, were added at various fivefold dilutions to each well (50 μl per well) and incubated overnight at 25°C. To the blocking MAb, 10 μl of the secondary ^{125}I-labeled MAb (approximately 2×10^5 cpm) was added and incubated for another 4 h at 37°C (34). After the second incubation, the contents of the wells were removed by aspiration and the wells were washed four times with PBS–0.05% Tween-20. Individual wells were counted in a gamma-counter (Clinigamma 1272; LKB-Wallac, Finland). MAbs inhibiting the binding of ^{125}I-labeled MAb by greater than 70% {percent inhibition = [1 − (amount of labeled MAb bound/amount of labeled MAb bound without blocking)] × 100} were considered to recognize the same or adjacent epitopes. All the ^{125}I-labeled MAbs were also tested against avirulent *S. flexneri* M90T-A_V, which does not contain the *ipa* genes, to determine the amount of nonspecific binding relative to the *S. flexneri* M90T-W positive control.

Construction of the λgt11 expression library from pHC17 DNA. Insert DNA for the λgt11HC17 expression library was derived from pHC17, a pBR322 recombinant containing a 4.75-kb *Hin*dIII fragment of the wild-type *S. flexneri* invasion plasmid pWR100 (M. Venkatesan, J. M. Buysse, and D. J. Kopecko, *Proc. Natl. Acad. Sci. USA*, in press). Plasmid pHC17 encoded the synthesis of complete IpaB and IpaC polypeptides as determined by immunoblot analysis of *E. coli* HB101(pHC17) cells and *E. coli* DS410(pHC17) minicells probed with rabbit screening antisera and the MAbs. pHC17 DNA was isolated as described previously (19) and individual samples of pHC17 DNA (3.2 μg) were digested to completion with one of six restriction enzymes (*Ava*II, *Dde*I, *Hin*fI, *Hin*PI, *Hpa*II, and *Taq*I). The digested pHC17 DNA was used to prepare adaptor-modified insert DNA by the procedure of Stover et al. (35) prior to cloning. Recombinant phage DNA was packaged into phage heads using an in vitro packaging system (Promega Biotech, Madison, Wis.) and were plated on *E. coli* Y1090 cells (*ΔlacU 169 proA*$^+$ *Δlon araD139 rpsL supF trpC*::Tn*10 hsdR hsdM*$^+$ *lacI*^{q}) (37) for screening. Recombinant phage produced colorless plaques on agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Screening and antigenic analysis of λgt11HC17 and λgt11Sfl expression libraries. Recombinant phage from the λgt11Sfl library, constructed previously in this laboratory (4), and the λgt11HC17 library were screened for antigen production in an immunoplaque assay using rabbit antisera specific for *S. flexneri* invasion plasmid antigens, as described by Young and Davis (38) and modified by Buysse et al. (4). To identify those clones encoding the synthesis of the IpaB and IpaC epitopes, purified λgt11Sfl and λgt11HC17 recombinants were screened with the IpaB- and IpaC-specific MAbs. One microliter of each recombinant phage (10^{10} PFU/ml) was spotted onto a lawn of Y1090 cells. After 2 to 3 h at 42°C, when the plaques became visible, nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) saturated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were placed on the plates and incubated at 37°C for 90 min. The nitrocellulose filters were then lifted from the plates and blocked with casein filler. The development of the filters with the MAbs was performed as described below.

Recombinant IpaB- and IpaC-expressing phage were used to prepare lysogens in Y1090 cells. The recombinant antigen epitopes synthesized by the lysogens were detected by immunoblot analysis as described previously (4), using the ^{125}I-labeled MAbs as probes.

Construction of a genetic map of the cloned λgt11 *ipaB* and *ipaC* epitopes. The mapping of insert DNA from λgt11Sfl recombinants has previously been described by workers in this laboratory (4). Phage DNA from the λgt11HC17 recombinants was obtained by the phenol extraction method of Silhavy et al. (32), and the size and number of insert fragments in each recombinant phage were determined by *Eco*RI digestion and analysis on 1.0% agarose gels. After separation on identically prepared agarose gels, recombinant DNA was analyzed by Southern blot hybridization (33) using a number of pHC17-derived DNA probes, including (i) pBR322, to identify hybrid vector fragments and fragments solely derived from pBR322, and (ii) the 1.4-kb *Hin*dIII-*Eco*RI, 2.5-kb *Eco*RI-*Eco*RI, and 0.9-kb *Eco*RI-*Hin*dIII fragments derived from the *ipaB* and *ipaC* encoding regions of pHC17 (see Fig. 2). All of the probes were purified from *Hin*dIII-*Eco*RI double-digested pHC17 DNA by electroelution and were labeled with [α-^{32}P]dCTP by nick-translation (nick-translation kit; New England Nuclear Corp., Boston, Mass.). Insert DNA from the λgt11HC17 recombinants was mapped more precisely using the purified insert fragments as probes against pHC17 and pWR100 DNA digested with various combinations of the restriction enzymes *Hin*dIII, *Pst*I, *Eco*RI, *Sal*I, and *Bgl*II and by cross-hybridization between the individual recombinants.

Plaque reduction assay. The effects of the MAbs on the invasive capacity of shigellae were determined by the plaque reduction assay. The plaque assay was performed as previ-

ously described (24) except that baby hamster kidney cells (BHK 21/15) were used instead of HeLa cells and the plaques were counted at 48 h postinoculation. The use of BHK cells improved the assay by increasing by about 100-fold the ability to detect invasive *Shigella* spp. A 4-h culture of *S. flexneri* M90T-W, grown at 37°C, was diluted 1:10,000 in sterile PBS and incubated with ammonium sulfate-precipitated MAb (final dilution 1:20) in sterile PBS. The mixture was incubated at 37°C for 45 min and then applied (0.2 ml to each dish) to the BHK cell monolayers. Five plaque dishes were set up for each antibody tested. The negative control antibody was an MAb against a trypanosome antigen. The number of plaques formed by the MAb-treated shigellae was compared with the negative control value.

RESULTS

Production of MAbs against the IpaB and IpaC polypeptides. Two spleen cell-myeloma cell fusions resulted in 283 wells of proliferating hybridoma cells, 12 of which contained cells producing antibodies against virulence-specific determinants. Immunoblot analysis of the positive culture supernatants revealed that five antibody-producing cultures (1H4, 2F1, 4C8, 5F4, and 6F4) synthesized antibodies reactive with the IpaB polypeptide, and seven cultures (4D4, 5H1, 5B1, 9B6, 2G2, 3G2, and 5F2) produced antibodies that recognized the IpaC polypeptide (Fig. 1; data for 3G2 and 6F4 not shown). Hybridomas from these 12 wells, producing virulence-specific antibodies, were cloned twice, and ascites fluids were prepared. The immunoglobulin subclass of most of the MAbs was IgG1, with the exception of 2G2, 3G2, and 2F1, which produced IgG2a monoclones.

Specificity of MAbs for virulent *Shigella* spp. and EIEC. The titer for each MAb was determined against both *S. flexneri* M90T-W and M90T-A_3 by a whole-cell ELISA. All of the MAbs had high titers against the virulent *S. flexneri* M90T-W, with little to no reactivity against the avirulent *S. flexneri* M90T-A_3 (Table 2). The complete set of MAbs were also used to screen other virulent and avirulent strains of *S. boydii*, *S. dysenteriae*, *S. flexneri*, *S. sonnei*, and EIEC by the whole-cell ELISA. All of the MAbs tested positive against all virulent strains, and none of the MAbs reacted with any of the avirulent strains (Table 1). To insure that there was no difference between the avirulent and virulent strains in their ability to adhere to the ELISA plate, both *S. flexneri* strains M90T-A_3 (avirulent) and M90T-W (virulent) were screened in an ELISA against culture supernatants from hybridoma lines (2A6 and 2C2) that produced antibodies which were not virulence specific. Immunoblot analysis with culture supernatants from 2A6 and 2C2 showed that they recognized a 70-kDa and a 45-kDa polypeptide, respectively. Both of these antigens were expressed by the avirulent and virulent strains of *S. flexneri*. For both 2A6 and 2C2 culture supernatants, the A_{405} values in the whole-cell ELISA did not differ between the avirulent and virulent strains (data not shown). Other enteric pathogens (i.e., enteropathogenic, enterotoxigenic, and hemorrhagic strains of *E. coli*; *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Salmonella* [28 species], *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Aeromonas hydrophila*) screened in the whole-cell ELISA did not react with any of the virulence-specific MAbs.

Epitope specificities of the MAbs. A competitive binding assay was used to identify the number of unique epitopes

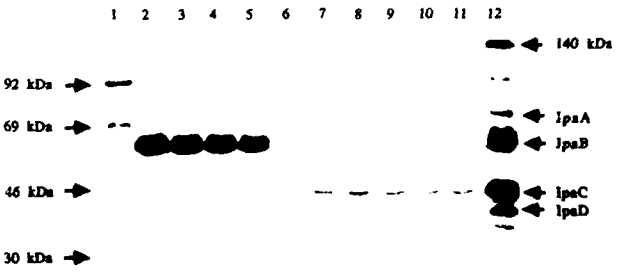

FIG. 1. Recognition of the IpaB or IpaC polypeptide by MAbs. ^{125}I-labeled MAbs and human convalescent antiserum were reacted with *S. flexneri* M90T-W whole-cell lysates that had been separated by SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose. (Lane 1) ^{14}C-labeled molecular weight markers (Amersham Corp.). (Lanes 2 through 5) Reacted with ^{125}I-labeled, IpaB-specific MAbs 1H4, 2F1, 4C8, and 5F4, respectively. (Lanes 6 through 11) Reacted with ^{125}I-labeled, IpaC-specific MAbs 2G2, 4D4, 5B1, 5H1, 5F2, and 9B6, respectively. (Lane 12) ^{125}I-labeled protein A was used to detect antibodies in the human convalescent antiserum against IpaA, IpaB, IpaC, IpaD, and the 140-kDa polypeptide.

recognized within each group of Ipa-specific MAbs. MAbs recognizing the same or closely adjacent epitopes were identified by their capacity to equally inhibit (by at least 70%) the binding of each other (Table 3). Three unique IpaB epitopes were defined by MAb 1H4, MAb 2F1, and the MAb trio of 4C8, 5F4, and 6F4. Similarly, MAb 5B1, MAb 9B6, the MAb pair 4D4 and 5H1, and the MAb trio of 2G2, 3G2, and 5F2 defined four unique IpaC epitopes (Table 3).

Plaque reduction assay. The strong correlation of the presence of polypeptides IpaB and IpaC with the invasive capacity of shigellae, and also the reactivity of the MAbs with intact virulent organisms in the ELISA, suggested that polypeptides IpaB and IpaC are located on the periphery of the bacterium and may be involved in the interaction with host cell membranes. By using the plaque assay, which is a more sensitive measure of invasion than light microscopy invasion assays, we determined whether shigellae pretreated

TABLE 2. Titers of IpaB- and IpaC-specific MAbs

MAb	Titer[a] with strain:	
	M90T-W	M90T-A_3
IpaB specific		
1H4	1,280	20
2F1	2,560	40
4C8	320	20
5F4	320	20
6F4	320	20
IpaC specific		
4D4	40,960	20
5H1	20,480	20
5B1	20,480	20
9B6	5,120	20
2G2	10,240	20
3G2	10,240	20
5F2	10,240	20

[a] Titers were determined in a whole-cell ELISA using both *S. flexneri* M90T-W (virulent) and *S. flexneri* M90T-2 (avirulent) as antigens. Ascites fluids of each MAb were serially diluted twofold starting from a 1:20 dilution. Alkaline phosphatase-conjugated goat anti-mouse IgG was used to detect the bound MAb. Endpoints given are reciprocals of the final dilution giving an A_{405} of ~0.3 within 60 min.

TABLE 3. Competitive binding assays with IpaB- and IpaC-specific MAbs

Blocking MAb	Binding of ^{125}I-labeled MAb (cpm)									
	IpaB specific				IpaC specific					
	1H4	2F1	4C8	5F4	4D4	5H1	5B1	9B6	2G2	5F2
IpaB specific										
1H4	204*	1,247	7,214	5,793	3,856	14,781	24,384	2,432	5,555	4,463
2F1	4,884	343*	6,148	5,356	3,180	14,032	25,599	2,741	5,785	4,534
4C8	3,838	1,260	250*	425*	3,297	14,328	24,231	2,384	5,616	4,865
5F4	5,151	1,178	255*	478*	3,577	14,569	23,503	2,316	6,482	4,213
6F4	5,055	1,215	232*	421*	3,448	13,078	23,751	2,339	5,594	4,752
IpaC specific										
4D4	4,682	1,221	5,698	5,601	292*	988*	26,703	2,815	4,590	2,854
5H1	4,892	1,367	6,127	5,803	489*	1,292*	27,214	2,405	5,099	3,561
5B1	4,790	1,345	6,776	5,396	4,816	18,999	770*	2,643	4,349	5,008
9B6	4,873	1,347	6,806	5,759	4,305	17,164	28,364	890*	5,502	4,869
2G2	4,844	1,323	6,102	5,314	3,209	12,860	23,010	2,261	289*	895*
3G2	4,792	1,408	4,808	5,449	3,215	11,497	22,263	2,011	287*	693*
5F2	4,847	1,339	5,672	5,465	3,219	13,099	22,133	2,091	212*	814*
Controls[b]										
Positive	5,496	1,257	6,847	5,146	4,293	19,501	22,848	3,445	6,709	4,352
Negative	403	253	441	276	95	857	466	1,315	466	103

[a] *S. flexneri* M90T-W was incubated with blocking MAb and then ^{125}I-labeled MAb was added. Each value given is the average counts per minute from duplicate wells and represents the amount of ^{125}I-labeled MAb that was able to bind. Significant reductions in the binding of ^{125}I-labeled MAb are denoted by an asterisk.

[b] The positive control was only incubated with ^{125}I-labeled MAb (i.e., no blocking MAb was used). Similarly, the negative control was also incubated with only ^{125}I-labeled MAb, except the avirulent strain M90T-A$_V$ was used as the antigen instead of M90T-W.

with IpaB- or IpaC-specific MAb had an altered capacity to form plaques in tissue culture cell monolayers. One of the IpaB-specific MAbs (2F1) reduced the plaque-forming capacity by more than 60%, whereas one of the IpaC-specific MAbs (2G2) enhanced the plaque-forming capacity by more than 50% (Table 4). The other IpaB- and IpaC-specific MAbs tested (which all recognized different epitopes based on competitive binding assays) had less of an effect on the plaque-forming capacity of *S. flexneri*.

Immunoplaque analysis of λgt11 recombinants with the MAbs. λgt11HC17 and λgt11Sfl recombinants reactive with the rabbit screening antisera were tested against the MAbs recognizing the seven unique epitopes of the IpaB and IpaC polypeptides. Recombinants expressing only one (λgt11 HC17-Sb1), two (λgt11HC17-Hr7), or all three (λgt11HC17-Tb1, λgt11Sfl-S43) of the epitopes recognized by the IpaB-specific MAbs were found (Fig. 2). Other recombinants encoding the synthesis of only one (λgt11HC17-Sc4), three (λgt11Sfl-S45), or all four (λgt11HC17-Dc1, λgt11Sfl-W12) of the epitopes recognized by the IpaC-specific MAbs were identified (Fig. 2). In addition, a recombinant (λgt11Sfl-S47) expressing epitopes reactive with all the MAbs except one IpaC epitope (2G2) was found. Recombinant phage such as λgt11Sfl-S17 that did not express epitopes recognized by any of the MAbs were also isolated, demonstrating that the rabbit antisera detected Ipa polypeptide epitopes that the MAbs did not recognize.

Genetic mapping of *ipaB* and *ipaC* epitopes. The map positions of the recombinants' insert DNA, plus the reactivities of the recombinants with the MAbs, allowed a genetic map of the *ipaB* and *ipaC* epitopes to be constructed. DNA hybridization studies defined the positions of the λgt11 insert fragment DNAs relative to the invasion plasmid pWR100 restriction map (4). Assuming that (i) the MAbs recognize epitopes encoded by a small contiguous DNA region, (ii) every recombinant reactive with a particular MAb carries the same epitope encoding DNA sequence, and (iii) that recombinants that do not react do not carry the corresponding sequences, then the region of DNA specifying the epitope sequence can be defined (Fig. 2).

Three IpaB epitopes were found to be encoded in a 700-base-pair (bp) DNA segment corresponding to the amino-terminal end of the IpaB polypeptide (Fig. 2) (see below). The amino-proximal 2F1 epitope constituted the largest region mapped (320 bp), with the 1H4 (240 bp) and 4C8 (140 bp) epitopes comprising the remaining 380 bp of the *ipaB* epitope regions. The amino-distal *ipaB* 4C8 epitope was separated from a cluster of three *ipaC* epitopes by 940 bp. These three *ipaC* epitopes were mapped within a 640-bp region corresponding to the amino-terminal end of the *ipaC* gene (see below). A fourth *ipaC* region containing the 2G2 epitope sequence (150 bp) was closely linked (~50 bp) to the IpaC amino-proximal cluster but was separable from these

TABLE 4. Effect of IpaB- and IpaC-specific MAbs on plaque formation

MAb	Expt no.	Avg PFU[a]	% of control[b]
IpaB specific			
1H4	1	64 ± 1.6	79.2
	2	344 ± 31.0	82.7
2F1	1	24 ± 4.0	29.3
	2	171 ± 9.8	40.6
4C8	1	50 ± 10.6	62.4
	2	271 ± 14.0	64.4
Control	1	81 ± 4.7	100.0
	2	421 ± 2.8	100.0
IpaC specific			
5H1	3	108 ± 9.6	136
5B1	3	67 ± 10.3	84.5
9B6	3	62 ± 6.1	77.3
2G2	3	124 ± 9.9	156
Control	3	80 ± 4.9	100.0

[a] The average PFU ± standard error was calculated from five plaque dishes.

[b] The percentage relates number of plaques formed by the Ipa-specific MAb-treated shigellae to the number of plaques formed by trypanosome-specific MAb-treated shigellae; the PFU in the latter was defined as 100% (i.e., control). The percentage was derived from the control of that particular experiment.

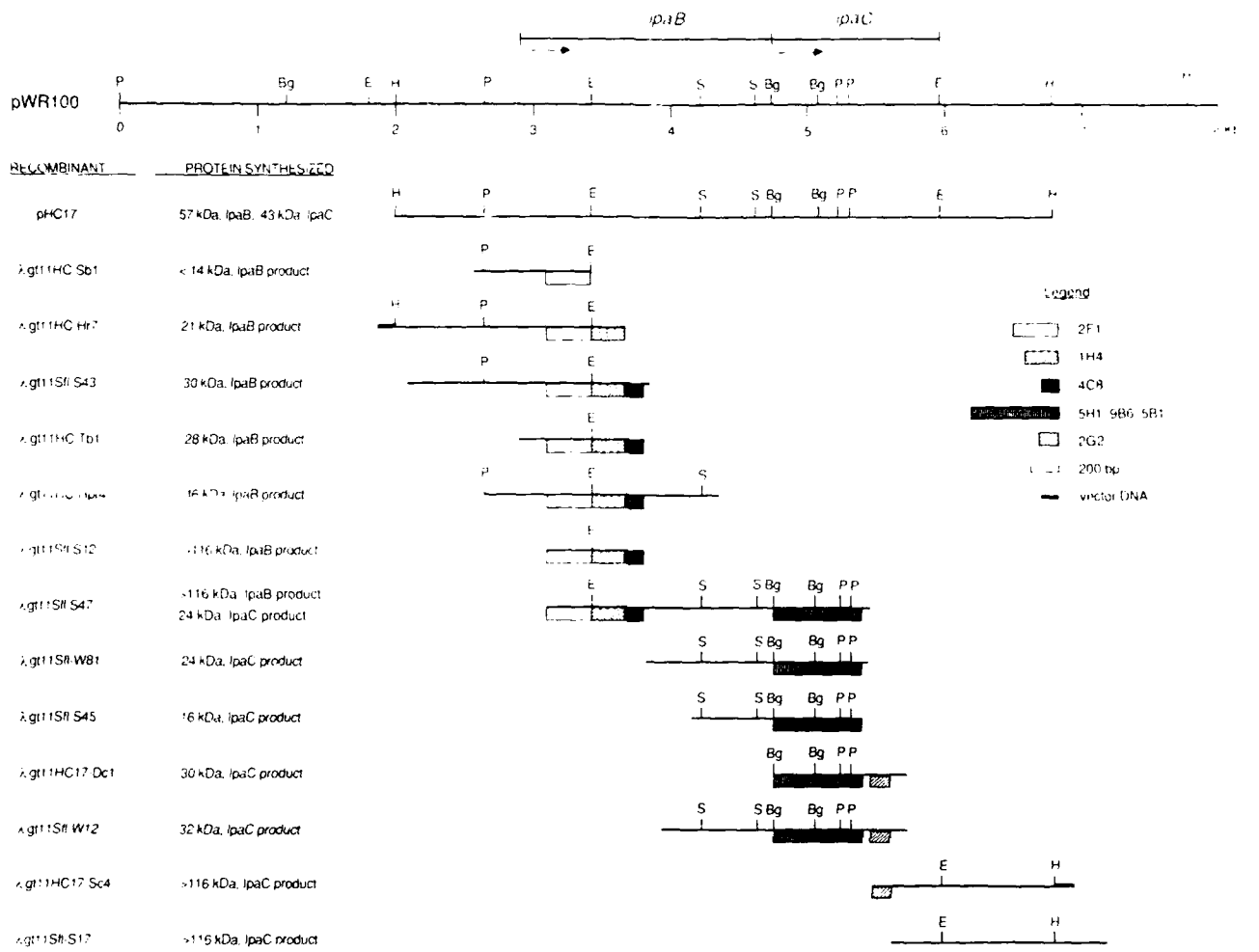

FIG. 2. Genetic map of the *ipaB* and *ipaC* epitopes. Hybridization analysis defined the positions of the λgt11 insert fragment DNAs relative to the invasion plasmid pWR100 restriction map (P, *Pst*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I). The maximal size of each epitope-encoding region is shown below every insert fragment DNA that encoded the epitope. The MAb epitope-encoding regions were defined based on the following assumptions: (i) the MAbs recognized epitopes encoded by small contiguous DNA regions; (ii) all recombinants reactive with an MAb carried the same epitope-encoding DNA sequence; and (iii) recombinants not reactive with an MAb do not carry the encoding sequence. The Ipa protein products synthesized by the recombinants were identified by immunoplaque analysis, and their molecular weights were determined by SDS-polyacrylamide gel electrophoresis analysis of lysogen lysates (see Fig. 3). Ipa protein products of >116 kDa required IPTG for induction of expression. The approximate locations of the *ipaB* and *ipaC* genes and their direction of transcription (denoted by arrows) are indicated above the pWR100 restriction map.

epitopes as shown by its isolation in λgt11 recombinants such as λgt11HC17-Sc4 (Fig. 2).

Ipa polypeptide products synthesized by λgt11 lysogens and determination of transcription polarity for the *ipaB* and *ipaC* genes. To determine the polarity of the epitopes with respect to the amino-terminal end of the IpaB or IpaC polypeptide, it was necessary to examine whole-cell lysates of Y1090::λgt11*ipaB* and Y1090::λgt11*ipaC* lysogens by SDS-polyacrylamide gel electrophoresis. Lysogens were found that encoded the synthesis of *lac*-dependent fusion polypeptides (i.e., >116 kDa) or *lac*-independent non-fusion polypeptides (i.e., IpaB products of ≤57 kDa or IpaC products of ≤43 kDa). The non-fusion polypeptides were independent of IPTG induction, suggesting that the insert DNA sequences provided the necessary transcriptional and translational start signals for the synthesis of the recombinant product. Strain Y1090::λgt11HC17-Sb1 synthesized a *lac*-independent, truncated (<14 kDa) IpaB product that only reacted with MAb 2F1 (Fig. 3). This finding indicated that the 2F1 epitope was nearest the amino-terminal end of the IpaB antigen (Fig. 2). Recombinants containing insert DNA that extended downstream of the 2F1 epitope-encoding region made proportionally larger peptides with additional epitope specificities. As an example, the Y1090::λgt11HC17-Hr7 lysogen expressed a non-fusion, truncated (21-kDa) IpaB product that reacted with MAbs 2F1 and 1H4, suggesting that the 1H4 epitope was adjacent to the 2F1 epitope. All of the IpaB-specific MAbs reacted with the truncated (28-kDa) IpaB product of Y1090::λgt11HC17-Tb1, demonstrating that epitope 4C8 was adjacent to the 1H4 epitope and nearest to the carboxyl terminus of the IpaB polypeptide. The translational start site for the *ipaB* gene was deduced by this analysis to be located between coordinates 2.9 and 3.1 (Fig. 2), since these sites define the ends of recombinants λgt11HC17-Tb1 and λgt11Sfl-S12, which en-

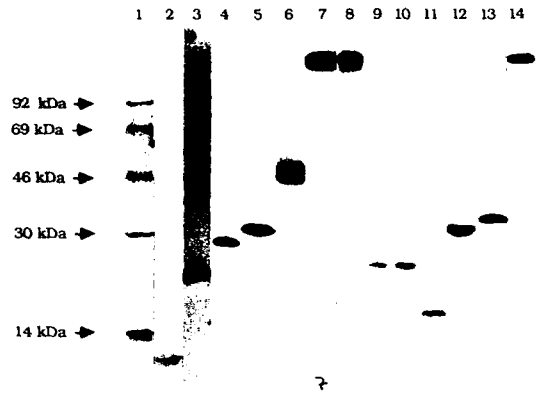

FIG. 3. Immunoblot analysis of Y1090::λgt11*ipaB* and *ipaC* lysogens probed with ^{125}I-labeled IpaB- or IpaC-specific MAbs. (Lanes 1) ^{14}C-labeled molecular weight markers (Amersham Corp.). (Lanes 2 through 14) Lysogen lysates in the following order (lane number): Sb1 (2), Hr7 (3), Tb1 (4), S43 (5), Hpr4 (6), S12 (7), S47 (8 and 9), W81 (10), S45 (11), Dc1 (12), W12 (13), and Sc4 (14). The recombinant products were detected with (lanes 2 through 8) IpaB-specific MAb 2F1, (lanes 9 through 13) IpaC-specific MAb 5B1, or (lanes 14) IpaC-specific MAb 2G2. Lysogens S47 (only for the IpaB product) and Sc4 required IPTG for induction of expression.

code a truncated polypeptide and *lac*-fusion polypeptide of the IpaB antigen, respectively.

λgt11*ipaC* recombinants were used in a similar analysis of the proteins synthesized by their corresponding lysogens. Recombinants λgt11Sfl-S47, W81, S45, and W12 were alike in that each contained insert DNA extending into portions of both the *ipaB* and *ipaC* genes (Fig. 2). However, the phenotypic expression of these recombinant molecules was not similar. Recombinant S47 expressed a *lac*-dependent fusion peptide carrying the three IpaB epitopes (2F1, 1H4, 4C8) and a *lac*-independent, 24-kDa truncated product of the *ipaC* gene harboring the 5B1, 5H1, 9B6 epitope cluster. In contrast, clones W81, S45, and W12 did not carry the insert DNA in the correct frame in relation to the *lacZ* promoter of λgt11 to synthesize fusion IpaB products, but they did synthesize various truncated IpaC products in the absence of IPTG, suggesting a unique set of transcriptional and translational initiators for IpaC that function independently of the IpaB initiators. The cluster of three IpaC epitopes (5B1, 5H1, 9B6) was found to be amino proximal on the IpaC antigen since Y1090::λgt11 recombinants S47, W81, and S45 gave proportionally smaller truncated peptides than recombinants W12 and Dc1, which also encoded the 2G2 epitope (Fig. 2). Y1090::λgt11 recombinants Sc4 and S17 produced fusion proteins lacking the amino-terminal cluster of three epitopes but carrying the 2G2 epitope (Sc4) or an additional epitope(s) recognized only by rabbit antisera and not by the MAbs (S17).

DISCUSSION

The interaction between facultative and obligate intracellular bacterial pathogens and host cells is a complex event that is accomplished by many different strategies (reviewed in reference 22). *Shigella* and *Rickettsia* spp. are among the only bacteria which replicate freely in the cytoplasm of host cells (22). To reach the cytoplasm, a bacterial surface-host cell surface interaction must take place which enables attachment to occur, followed by an "induced phagocytosis" and eventual escape of the bacterium from the phagocytic vacuole. For shigella the escape process appears to be mediated by the bacterium (29). The multistep process of invasion most likely involves several bacterial products, a situation vastly different from the one gene required for the invasive phenotype of *Y. pseudotuberculosis* (12). To date, the minimum set of genes required for the invasive phenotype in *Shigella* spp. has not been identified. Genes on the 120- to 140-MDa plasmid within a 37-kb region are necessary for invasion, with the *ipa* genes being the most promising candidates, but it has not yet been possible to isolate the *ipa* genes by a combination of mutation and complementation to prove their role in invasion.

The individual *ipaB* and *ipaC* gene products have not been studied in great detail largely because of their extreme sensitivity to proteolysis (E. V. Oaks, unpublished observations) and relative low quantities in the organism (8). To facilitate the biochemical and functional analyses of the IpaB and IpaC polypeptides, we have produced and used MAbs to isolate the IpaB and IpaC polypeptides for study. Previously, investigations have indicated that IpaB and IpaC are associated with the cell envelope (9) and are most likely present on the surface of shigellae. The ability to extract these polypeptides with mild procedures such as water extraction (23), and the reactivity of virulent-specific polyvalent rabbit sera (reactive with both IpaB and IpaC) with whole organisms in an ELISA (25; T. Pal, personal communication), lend support to a surface exposure model. In this study the reactivity of all the IpaB- and IpaC-specific MAbs with whole organisms in an ELISA has demonstrated clearly that both IpaB and IpaC are exposed on the surface of virulent shigellae. In addition, the three IpaB and four IpaC surface epitopes have been shown to be highly conserved among *Shigella* serotypes and EIEC (Table 1). Cross-reactive epitopes were not found on other enteric organisms, including invasive organisms such as *Salmonella*, *Yersinia*, and *Campylobacter* spp. This supports earlier studies that showed the uniqueness of the *ipa* genes to virulent *Shigella* and EIEC strains, using *ipa* gene probes (36), and also indicates that the MAbs could be used as diagnostic probes to specifically identify virulent *Shigella* and EIEC isolates. The conservation of epitopes in a surface structure which is very likely exposed to many environmental stresses suggests that these polypeptides are essential to the virulent phenotype and that mutations or alterations in these structures may result in virulence attenuation.

The ability of one IpaB MAb (2F1) to reduce plaque formation lends additional support to the notion that the IpaB polypeptide may be actively involved in the invasion process. The exact step (i.e., attachment, endocytosis, escape from the phagosome, replication within cells) which is being blocked by the MAb could not be determined by the plaque reduction assay. Other assays, such as light microscopy examination of host cell invasion by antibody-treated shigellae, which might be used to pinpoint the step of inhibition, do not have the sensitivity of the plaque assay, in that they require very heavy burdens (10^8) of shigellae and mechanical assistance (centrifugation) to achieve a moderate infection (6). Such high numbers of organisms would be very difficult to block with antibodies. The inability of other IpaB-specific MAbs to inhibit plaque formation (at a level comparable to 2F1) indicates that other regions of the IpaB polypeptide are not as crucial to invasion as the 2F1 epitope region. None of the IpaC-specific MAbs tested in the plaque reduction assay was inhibitory, although one IpaC MAb (2G2) actually enhanced the plaque-forming capacity of *S.*

flexneri by 50%. Using polyclonal sera, earlier studies by Hale and Bonventre also demonstrated an enhancing effect of antibodies (6). Enhancement may occur due to an altered conformation of the IpaB protein that, either directly or indirectly, allows the invasion process to occur in a more efficient manner. For either enhancement or inhibition, it appears that the invasive process can be altered by antibody against certain epitopes of the IpaB and IpaC polypeptides.

Recent advances in the λgt11 cloning system (38) have permitted the cloning and expression of small DNA segments representing portions of a protein as small as a single epitope (35). By using this approach to clone the DNA segments encoding epitopes, an epitope map was constructed of IpaB and IpaC, representing the structural genes, the translational orientation, and the approximate location of each of the seven unique epitopes. The three IpaB epitopes (2F1, 1H4, and 4C8) were all expressed on a 28-kDa peptide (expressed by λgt11HC-Tb1). An intermediate-sized peptide of 21 kDa (expressed by λgt11HC17-Hr7) contained two epitopes (2F1 and 1H4), and a smaller peptide less than 14 kDa (synthesized by λgt11HC17-Sb1) expressed only the 2F1 epitope. Assuming that the small peptides represent truncated versions of the IpaB polypeptide that were synthesized from a DNA insert containing the regulatory elements (i.e., promoter, ribosome binding sites, etc.) as well as a portion of the structural gene, then it is possible to place the epitopes at the amino-terminal half of the IpaB protein, with epitope 2F1 being closest to the amino terminus.

A cluster of three unique IpaC epitopes (5H1, 5B1, and 9B6) were expressed at the amino-terminal half of the IpaC protein. Unlike the three IpaB-specific MAbs, which all show unique reactivities with the various *ipaB* recombinants, it was not possible to orient the 5H1, 5B1, 9B6 cluster within the IpaC polypeptide except that a 16-kDa truncated peptide (expressed by λgt11Sfl-S45) contained all three epitopes. A 30-kDa peptide expressed by lysogen Y1090::HC17-Dc1 contained all four IpaC epitopes, including the epitope recognized by MAb 2G2. Another recombinant (λgt11HC17-Sc4), expressing only epitope 2G2 as a fusion protein, contained DNA which mapped downstream of the 5H1, 5B1, 9B6 cluster. Based on the approximate gene sizes for the IpaB and IpaC polypeptides, the mapping data suggest that potentially large regions of the IpaB and IpaC polypeptides are exposed on the surface of *Shigella* spp. In fact, DNA sequence data indicate that both the IpaB and IpaC polypeptides have long hydrophilic stretches corresponding to the epitope locations (Venkatesan et al., in press).

The epitope map (Fig. 2) was based on the assumption that the epitopes are linear (continuous). This assumption allows the epitopes to be defined to unique linear segments on the basis of exclusion of DNA segments not expressing the epitopes. However, in certain cases, conformational (discontinuous) epitopes cannot be ruled out because a small DNA segment expressing a unique epitope was not isolated. For example, IpaB epitope 4C8 is represented as a 0.24-kb DNA fragment, assuming the epitope is linear, but it is also possible to consider 4C8 as a conformational epitope requiring the entire 0.58-kb region in *ipaB* from map position 3.1 to ~3.7. By isolating new recombinants expressing only the 4C8 epitope and not 2F1 and 1H4, or by using the DNA sequence of *ipaB* to generate peptides through the epitope-encoding region, it can be determined whether the 4C8 epitope is linear or conformational. Either way, λgt11 cloning has permitted the localization of these epitopes to very small regions of the corresponding genes, in lieu of DNA sequence or synthetic peptide information.

The surface location of the amino-terminal IpaB and IpaC epitopes suggests that at least these regions of the IpaB and IpaC polypeptides are hydrophilic and possibly very immunogenic. Previous studies by Oaks et al. (23) have demonstrated that the dominant antibody response in human and monkey convalescent sera is against IpaB and IpaC, and it appears likely from the work of Dinari et al. (5) that intestinal secretions may also contain antibodies against IpaB and IpaC. The effect these antibodies have in vivo is still open to speculation, but it is possible that antibodies against IpaB and IpaC may inhibit invasion or at least opsonize virulent shigellae in preparation for destruction by complement or professional phagocytes. Further studies on defining the functional roles of these epitopes more precisely should advance our understanding of the invasive process as performed by *S. flexneri* and the subsequent host immune response against this pathogen.

ACKNOWLEDGMENTS

We thank S. Formal, P. Gemski, P. Guerry, D. Kopecko, F. Rubin, R. B. Sack, and M. Venkatesan for their help in obtaining bacterial strains; P. Echeverria for convalescent human antisera; T. L. Hale for HeLa cells and convalescent monkey antisera; T. Hall for X63-Ag8.653 myeloma cells and trypanosome-specific MAbs; and J. McCown for BHK 21/15 cells. A special acknowledgment goes to P. Rathod for his support and encouragement during the course of this research.

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